

We claim:

1. A modified pneumolysin polypeptide having attenuated hemolytic activity wherein said modified pneumolysin polypeptide is obtained by:
 - 5 a) randomly mutating a nucleic acid molecule encoding for wild-type pneumolysin to produce mutated nucleic acid molecules encoding modified pneumolysin polypeptides and expressing the mutated nucleic acid
10 molecules in host cells;
 - b) assaying the modified polypeptide expressed by the host cells for hemolytic activity;
 - 15 c) identifying the modified pneumolysin polypeptides having substantially similar molecular weight as native wild-type pneumolysin and which are refoldable.
2. A modified properly-refolded pneumolysin polypeptide having attenuated hemolytic activity comprising an amino acid sequence of type 14 pneumolysin wherein at
20 least one amino acid in the region comprising amino acid residues 1 to 257 is substituted and wherein at least one of said amino acid substitutions results in attenuation of the hemolytic activity of the modified pneumolysin polypeptide.
- 25 . The modified pneumolysin polypeptide of claim 2, wherein the hemolytic activity is less than 25% compared to wild-type pneumolysin.
4. A modified pneumolysin polypeptide according to claim 3, comprising at least one amino acid substitution in
30 the amino acid sequence of Formula I at residue positions 61, 148, or 195 or the combination of substitutions at residue positions 33, 46, 83, 239 and 257,

(Formula I)

| | | | | | | | | | | | | |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| | Met | Ala | Asn | Lys | Ala | Val | Asn | Asp | Phe | Ile | Leu | Ala |
| | 1 | | | | 5 | | | | | 10 | | |
| 5 | Met | Asn | Tyr | Asp | Lys | Lys | Lys | Leu | Leu | Thr | His | Gln |
| | | | 15 | | | | | 20 | | | | |
| | Gly | Glu | Ser | Ile | Glu | Asn | Arg | Phe | Ile | Lys | Glu | Gly |
| | 25 | | | | | 30 | | | | | 35 | |
| | Asn | Gln | Leu | Pro | Asp | Glu | Phe | Val | Val | Ile | Glu | Arg |
| 10 | | | | 40 | | | | | 45 | | | |
| | Lys | Lys | Arg | Ser | Leu | Ser | Thr | Asn | Thr | Ser | Asp | Ile |
| | | 50 | | | | | 55 | | | | | 60 |
| | Ser | Val | Thr | Ala | Thr | Asn | Asp | Ser | Arg | Leu | Tyr | Pro |
| | | | | 65 | | | | | | 70 | | |
| 15 | Gly | Ala | Leu | Leu | Val | Val | Asp | Glu | Thr | Leu | Leu | Glu |
| | | | 75 | | | | 80 | | | | | |
| | Asn | Asn | Pro | Thr | Leu | Leu | Ala | Val | Asp | Arg | Ala | Pro |
| | 85 | | | | | 90 | | | | | 95 | |
| | Met | Thr | Tyr | Ser | Ile | Asp | Leu | Pro | Gly | Leu | Ala | Ser |
| 20 | | | | 100 | | | | | 105 | | | |
| | Ser | Asp | Ser | Phe | Leu | Gln | Val | Glu | Asp | Pro | Ser | Asn |
| | | 110 | | | | | 115 | | | | | 120 |
| | Ser | Ser | Val | Arg | Gly | Ala | Val | Asn | Asp | Leu | Leu | Ala |
| | | | | | 125 | | | | | 130 | | |
| 25 | Lys | Trp | His | Gln | Asp | Tyr | Gly | Gln | Val | Asn | Asn | Val |
| | | | 135 | | | | | 140 | | | | |
| | Pro | Ala | Arg | Met | Gln | Tyr | Glu | Lys | Ile | Thr | Ala | His |
| | 145 | | | | | 150 | | | | | 155 | |
| | Ser | Met | Glu | Gln | Leu | Lys | Val | Lys | Phe | Gly | Ser | Asp |
| 30 | | | | 160 | | | | | 165 | | | |
| | Phe | Glu | Lys | Thr | Gly | Asn | Ser | Leu | Asp | Ile | Asp | Phe |
| | | 170 | | | | | 175 | | | | | 180 |
| | Asn | Ser | Val | His | Ser | Gly | Glu | Lys | Gln | Ile | Gln | Ile |
| | | | | 185 | | | | | | 190 | | |
| 35 | Val | Asn | Phe | Lys | Gln | Ile | Tyr | Tyr | Thr | Val | Ser | Val |
| | | | 195 | | | | | 200 | | | | |
| | Asp | Ala | Val | Lys | Asn | Pro | Gly | Asp | Val | Phe | Gln | Asp |
| | 205 | | | | | 210 | | | | | 215 | |
| | Thr | Val | Thr | Val | Glu | Asp | Leu | Lys | Gln | Arg | Gly | Ile |
| 40 | | | | 220 | | | | | 225 | | | |
| | Ser | Ala | Glu | Arg | Pro | Leu | Val | Tyr | Ile | Ser | Ser | Val |
| | | 230 | | | | | 235 | | | | | 240 |
| | Ala | Tyr | Gly | Arg | Gln | Val | Tyr | Leu | Lys | Leu | Glu | Thr |
| | | | | 245 | | | | | | 250 | | |
| 45 | Thr | Ser | Lys | Ser | Asp | Glu | Val | Glu | Ala | Ala | Phe | Glu |
| | | | 255 | | | | | 260 | | | | |
| | Ala | Leu | Ile | Lys | Gly | Val | Lys | Val | Ala | Pro | Gln | Thr |
| | 265 | | | | 270 | | | | | | 275 | |

| | | | | | | | | | | | | |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| | Glu | Trp | Lys | Gln | Ile | Leu | Asp | Asn | Thr | Glu | Val | Lys |
| | | | | 280 | | | | | 285 | | | |
| | Ala | Val | Ile | Leu | Gly | Gly | Asp | Pro | Ser | Ser | Gly | Ala |
| | 290 | | | | | | 295 | | | | | 300 |
| 5 | Arg | Val | Val | Thr | Gly | Lys | Val | Asp | Met | Val | Glu | Asp |
| | | | | | 305 | | | | | 310 | | |
| | Leu | Ile | Gln | Glu | Gly | Ser | Arg | Phe | Thr | Ala | Asp | His |
| | | | 315 | | | | | 320 | | | | |
| | Pro | Gly | Leu | Pro | Ile | Ser | Tyr | Thr | Thr | Ser | Phe | Leu |
| 10 | 325 | | | | | 330 | | | | | 335 | |
| | Arg | Asp | Asn | Val | Val | Ala | Thr | Phe | Gln | Asn | Ser | Thr |
| | | | | 340 | | | | | 345 | | | |
| | Asp | Tyr | Val | Glu | Thr | Lys | Val | Thr | Ala | Tyr | Arg | Asn |
| | 350 | | | | | | 355 | | | | | 360 |
| 15 | Gly | Asp | Leu | Leu | Leu | Asp | His | Ser | Gly | Ala | Tyr | Val |
| | | | | | 365 | | | | | 370 | | |
| | Ala | Gln | Tyr | Tyr | Ile | Thr | Trp | Asn | Glu | Leu | Ser | Tyr |
| | | | 375 | | | | | 380 | | | | |
| | Asp | His | Gln | Gly | Lys | Glu | Val | Leu | Thr | Pro | Lys | Ala |
| 20 | 385 | | | | | 390 | | | | | 395 | |
| | Trp | Asp | Arg | Asn | Gly | Gln | Asp | Leu | Thr | Ala | His | Phe |
| | | | | 400 | | | | | 405 | | | |
| | Thr | Thr | Ser | Ile | Pro | Leu | Lys | Gly | Asn | Val | Arg | Asn |
| | 410 | | | | | | 415 | | | | | 420 |
| 25 | Leu | Ser | Val | Lys | Ile | Arg | Glu | Cys | Thr | Gly | Leu | Ala |
| | | | | | 425 | | | | | 430 | | |
| | Trp | Glu | Trp | Trp | Arg | Thr | Val | Tyr | Glu | Lys | Thr | Asp |
| | | | 435 | | | | | 440 | | | | |
| | Leu | Pro | Leu | Val | Arg | Lys | Arg | Thr | Ile | Ser | Ile | Trp |
| 30 | 445 | | | | | 450 | | | | | 455 | |
| | Gly | Thr | Thr | Leu | Tyr | Pro | Gln | Val | Glu | Asp | Lys | Val |
| | | | | 460 | | | | | 465 | | | |
| | Glu | Asn | Asp | | | | | | | | | |
| | | | | 470 | | | | | | | | |

35 5. The modified pneumolysin according to claim 4,
 wherein a single amino acid substitution is made and
 the substituted amino acid is selected from the group
 consisting of proline or hydroxyproline for position
 61; lysine, arginine or histidine for position 148
 40 and leucine, glycine, alanine, isoleucine or valine
 for position 195.

6. The modified pneumolysin according to claim 3,
 wherein the substituted amino acids are selected from

- the group consisting of serine, threonine, asparagine, glutamine, tyrosine or [cystine] cysteine for positions 33, 46 and 83; lysine, arginine or histidine for position 239 and leucine, glycine, alanine, isoleucine or valine for position 255.
- 5
7. Modified pneumolysin polypeptide pNVJ1.
8. Modified pneumolysin polypeptide pNVJ20
9. Modified pneumolysin polypeptide pNVJ22.
10. Modified pneumolysin polypeptide pNVJ45.
- 10 11. Modified pneumolysin polypeptide pNVJ56.
12. Modified pneumolysin polypeptide pNV103.
13. Modified pneumolysin polypeptide pNV207.
14. Modified pneumolysin polypeptide pNV111.
15. Modified pneumolysin polypeptide pNV211.
- 15 16. A recombinant nucleic acid molecule encoding a modified type 14 pneumolysin polypeptide wherein at least one amino acid in the region comprising amino acid residues 1 to 257 is substituted and wherein at least one of said amino acid substitutions results in
- 20 attenuation of the hemolytic activity of the modified pneumolysin polypeptide.
17. The recombinant nucleic acid molecule according to claim 16 comprising the following pneumolysin nucleic acid sequence:
- 25 ATGGCAAATA AAGCAGTAAA TGACTTTATA CTAGCTATGA 40
ATTACGATAA AAAGAACTC TTGACCCATC AGGGAGAAAG 80

| | | | | | |
|----|-------------|-------------|------------|------------|------|
| | TATTGAAAAT | CGTTTCATCA | AAGAGGGTAA | TCAGCTACCC | 120 |
| | GATGAGTTTG | TTGTTATCGA | AAGAAAGAAG | CGGAGCTTGT | 160 |
| | CGACAAATAC | AAGTGATATT | TCTGTAACAG | CTACCAACGA | 200 |
| | CAGTCGCCTC | TATCCTGGAG | CACTTCTCGT | AGTGGATGAG | 240 |
| 5 | ACCTTGTTAG | AGAATAATCC | CACTCTTCTT | GCGGTCGATC | 280 |
| | GTGCTCCGAT | GACTTATAGT | ATTGATTTGC | CTGGTTTGGC | 320 |
| | AAGTAGCGAT | AGCTTTCTCC | AAGTGGAAGA | TCCCAGCAAT | 360 |
| | TCAAGTGTTT | GCGGAGCGGT | AAACGATTTG | TTGGCTAAGT | 400 |
| | GGCATCAAGA | TTATGGTCAG | GTCAATAATG | TCCCAGCTAG | 440 |
| 10 | AATGCAGTAT | GAAAAAATCA | CGGCTCACAG | CATGGAACAA | 480 |
| | CTCAAGGTCA | AGTTTGGTTC | TGACTTTGAA | AAGACAGGGA | 520 |
| | ATTCTCTTGA | TATTGATTTT | AACTCTGTCC | ATTCAGGCGA | 560 |
| | AAAGCAGATT | CAGATTGTTA | ATTTTAAGCA | GATTTATTAT | 600 |
| | ACAGTCAGCG | TAGACGCTGT | TAAAAATCCA | GGAGATGTGT | 640 |
| 15 | TTCAAGATAC | TGTAACGGTA | GAGGATTTAA | AACAGAGAGG | 680 |
| | AATTTCTGCA | GAGCGTCCTT | TGGTCTATAT | TTCGAGTGTT | 720 |
| | GCTTATGGGC | GCCAAGTCTA | TCTCAAGTTG | GAAACCACGA | 760 |
| | GTAAGAGTGA | TGAAGTAGAG | GCTGCTTTTG | AAGCTTTGAT | 800 |
| | AAAAGGAGTC | AAGGTAGCTC | CTCAGACAGA | GTGGAAGCAG | 840 |
| 20 | ATTTTGACACA | ATACAGAAGT | GAAGGCGGTT | ATTTTAGGGG | 880 |
| | GCGACCCAAG | TTCGGGTGCC | CGAGTTGTAA | CAGGCAAGGT | 920 |
| | GGATATGGTA | GAGGACTTGA | TTCAAGAAGG | CAGTCGCTTT | 960 |
| | ACAGCAGATC | ATCCAGGCTT | GCCGATTTCC | TATACAACTT | 1000 |
| | CTTTTTTTACG | TGACAATGTA | GTTGCGACCT | TTCAAAATAG | 1040 |
| 25 | TACAGACTAT | GTTGAGACTA | AGGTTACAGC | TTACAGAAAC | 1080 |
| | GGAGATTTAC | TGCTGGATCA | TAGTGGTGCC | TATGTTGCCC | 1120 |
| | AATATTATAT | TACTTGGAAT | GAATTATCCT | ATGATCATCA | 1160 |
| | AGGTAAGGAA | GTCTTGACTC | CTAAGGCTTG | GGACAGAAAT | 1200 |
| | GGGCAGGATT | TAACGGCTCA | CTTTACCACT | AGTATTCCTT | 1240 |
| 30 | TAAAAGGGAA | TGTTCTGTAAT | CTCTCTGTCA | AAATTAGAGA | 1280 |
| | GTGTACCGGG | CTTGCTTGGG | AATGGTGCGG | TACGGTTTAT | 1320 |
| | GAAAAAACCG | ATTTGCCACT | AGTGCGTAAG | CGGACGATTT | 1360 |
| | CTATTTGGGG | AACAACCTCTC | TATCCGCAGG | TAGAAGATAA | 1400 |
| | GGTAGAAAAT | GAC | | | 1413 |

35 and wherein said nucleic acid sequence comprises one or more of the nucleotide substitutions selected from the group consisting of:

A-50→G, G-54→T, T-181→C, A-196→T and T-302→C;

40 A-122→G, A-514→G, T-583→A and A-764→G;

A-187→T, T-380→A, A-382→C and T-443→A;

T-98→C, T-137→C, T-248→C, T-717→A and A-770→G;

T-134→C, A-305→G, A-566→G and T-583→G;

T-583→G;

5

T-583→A;

T-443→A;

and

10

T-181→C.

18. The recombinant nucleic acid molecule of claim 16 as contained in a vector such as a plasmid, cosmid, bacteriophage or yeast artificial chromosome.

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19. A microorganism comprising the nucleic acid molecule of claim 16.

20. The microorganism according to claim 19, wherein the microorganism is selected from the group consisting of bacteria, yeast, mammalian or insect cells.

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21. The microorganism according to claim 20, wherein the microorganism is *E. coli*.

22. The modified pneumolysin polypeptide of claim 1, wherein the polypeptide is conjugated to a polysaccharide which elicits antibodies cross-reactive with a bacterial polysaccharide.

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23. The modified pneumolysin conjugate of claim 22, wherein the polysaccharide is from a bacteria selected from the group consisting of a Haemophilus influenzae type b; meningococcal group A, B or C; group B streptococcus types Ia, Ib, II, III, V or

VIII and pneumococcal.

24. A vaccine comprising at least one pneumolysin polypeptide of claim 1 and a pharmaceutically acceptable carrier.
- 5 25. The vaccine according to claim 24, wherein the polypeptide is conjugated to a polysaccharide which elicits antibodies cross-reactive with a bacterial polysaccharide.
- 10 26. The vaccine according to claim 25, wherein the polysaccharide is derived from a bacteria selected from the group consisting of Haemophilus influenzae type b; meningococcus group A, B, or C; group A streptococcus or group B streptococcus serotypes Ia, Ib, II, III, V, or VIII; or one or more of serotypes 15 1-23 of *S. pneumoniae*.
27. A method for killing bacteria comprising contacting said bacteria with antibodies to an immunogenic molecule comprising the modified pneumolysin according to claim 1 in the presence of complement.
- 20 28. The method according to claim 27, wherein the immunogenic molecule is a polysaccharide-polypeptide conjugate wherein the polysaccharide is a bacterial capsular polysaccharide.
- 25 29. A method for immunization of mammals comprising administering the vaccine of claim 24 to said mammals.
30. A method for obtaining modified pneumolysin polypeptides having reduced hemolytic activity and

being suitable for eliciting an immunogenetic response which is cross-reactive with wild-type pneumolysin comprising the steps of:

- 5 a) randomly mutating a nucleic acid molecule
 encoding for wild-type pneumolysin to
 produce mutated nucleic acid molecules
 encoding modified pneumolysin polypeptides
 and expressing the mutated nucleic acid
 molecules in host cells;
- 10 b) assaying the modified polypeptide expressed
 by the host cells for hemolytic activity;
- c) identifying the modified pneumolysin
 polypeptides having substantially similar
 molecular weight as native wild-type
15 pneumolysin and which are refoldable.